Dendritic Cells Play a Crucial Role in Innate Immunity to Simple Chemicals

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Recently, it has been demonstrated that immunity to infectious agents is composed of innate immunity and acquired immunity, and that dendritic cells (DC) and macrophages, both of which are the participants in the innate immunity, play a crucial role in acquired immune responses, via their expression of several costimulatory molecules and production of cytokines. It is clear that the immune system responds not only to infectious organisms but also to simple chemicals. Allergic contact hypersensitivity reaction is a good example of the immune response to simple chemicals. In contrast to the immunity to microorganisms, however, the role of the innate immune system in responses to simple chemicals still remains unclear. This paper demonstrates that the activation and apoptosis of DC are directly induced by certain simple chemicals, and we suggest that DC, as cells involved in the innate immune system, play a crucial role in the immunity to simple chemicals. *Key words: contact hypersensitivity/costimulatory molecules/hapten/innate immunity. Journal of Investigative Dermatology Symposium Proceedings 4:158–163, 1999*

Two principal systems of immunity to infectious agents have been selected during evolution: (a) innate or natural immunity, and (b) acquired (adaptive) or specific immunity (**Fig 1**) (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997; Janeway, 1998). Innate immunity is phylogenetically older than acquired immunity. The essential difference between the two systems is the means by which they recognize microorganisms. The innate immunity system uses proteins encoded in the germ line to identify potentially noxious substances. These proteins usually seem to recognize carbohydrate structures. For example, macrophages endocytose particles or soluble glycoconjugates that are bound by the mannose receptor, a C-type lectin with broad carbohydrate specificity. In addition, they also have a receptor for lipopolysaccharide. On the other hand, using products of the RAG1 and RAG2 genes, B and T lymphocytes somatically rearrange the V, D, and J elements of their immunoglobulin and T cell receptor genes, respectively, to create as many as 10^{11} different clones of B or T lymphocytes that express distinct antigen receptors. Therefore, the innate immune system is inflexible in its recognition of targets, whereas the acquired immune system of lymphocytes is almost infinitely adaptable.

Using this highly adaptable system, however, acquired immunity has sacrificed a cardinal characteristic of innate immunity; the inherent ability to distinguish between potential pathogens that require an immune response and innocuous substances for which an immune response is either unnecessary or, in the case of self-antigen, rather injurious. Here innate immunity provides instruction that enables the acquired immune response to select appropriate antigens and eliminate them

For example, in the two-signal model of T cell activation (Kaye, 1995), T cell receptor ligation by major histocompatibility complex (MHC)-antigen provides signal 1, which is a primary activation signal mediated via the activation of protein tyrosine kinases, requiring the CD45 protein tyrosine phosphatase. Signal 2 is achieved by the interaction of a variety of receptor-counter receptor pairs, of which the best characterized are those involving CD80/CD86 and CD28/CTLA-4, which utilize biochemical pathways distinct from those initiated by the T cell receptor (TCR). The immunoregulatory potential of costimulation becomes clear when it is shown that TCR ligation in the absence of signal 2 could lead to T cell nonresponsiveness or anergy. This signal 2 is supplied by innate immunity, including various antigen-presenting cells (APC).

Among the various APC, dendritic cells (DC) are the most potent ones for helper T cells as well as cytotoxic T cells (Steinman, 1991). After their development in the bone marrow, they transiently reside in nonlymphoid organs where they are extremely active in endocytosis as well as in antigen processing (Romani *et al*, 1989a). In response to some stimuli derived from microorganisms, such as lipopolysaccharide (LPS) (Verhasselt *et al*, 1997), these precursor DC migrate to T cell zones of lymph nodes (Macatonia *et al*, 1987; Kripke *et al*, 1990) and mature into effective APC by ceasing endocytosis as well as their processing function, stabilizing expression of class II-peptide complexes (Kampgen *et al*, 1991; Stossel *et al*, 1990), and increasing expression of CD80 or CD86 (Romani *et al*, 1989b; Symington *et al*, 1993; Girolomoni *et al*, 1994; Ozawa *et al*, 1996). Other than LPS, DC have been reported to be activated by some bacteria like *Staphylococcus aureus* (Heufler *et al*, 1996) and *Mycobacterium tuberculosis* (Henderson *et al*, 1997) via an unknown mechanism, and by phagocytosis of microparticle-adsorbed protein (Scheicher *et al*, 1995), or apoptotic bodies (Rubartelli *et al*, 1997). These studies clearly demonstrated the crucial role played by DC in innate immunity to microorganisms.

Other than immunity to microorganisms, however, the immune system also responds to simple chemicals. Allergic contact hypersensitivity reaction is a good example of the immune response to simple chemicals. In this paper, we demonstrate that the activation and apoptosis of DC are directly induced by some restricted simple

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Abbreviations: BC, benzalkonium chloride; DC, dendritic cell; DNCB, dinitrochlorobenzene; PBMC, peripheral blood mononuclear cells; SLS, sodium lauryl sulfate; TNCB, trinitrochlorobenzene.

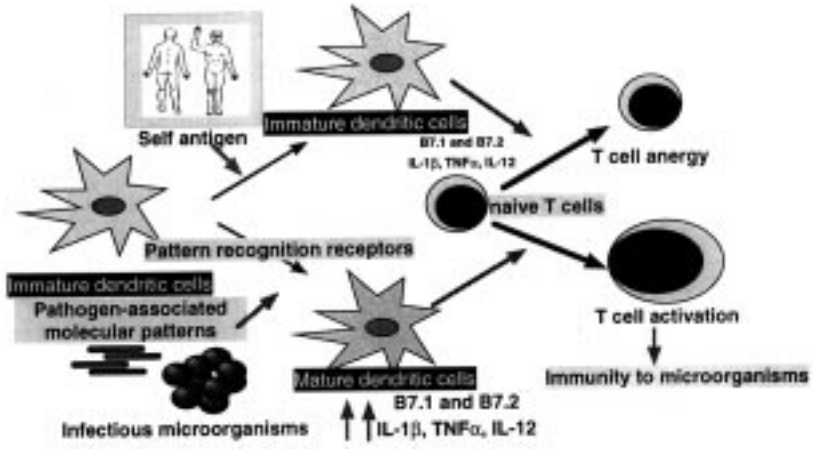

Figure 1. The instructive role of innate immunity played by DC in the acquired immune response to infectious agents. Immature DC are stimulated by infectious microorganisms via pattern-recognition receptors, and augment their expression of costimulatory molecules and secretion of pro-inflammatory cytokines. These mature DC can stimulate naïve T cells and induce specific immunity to microorganisms.

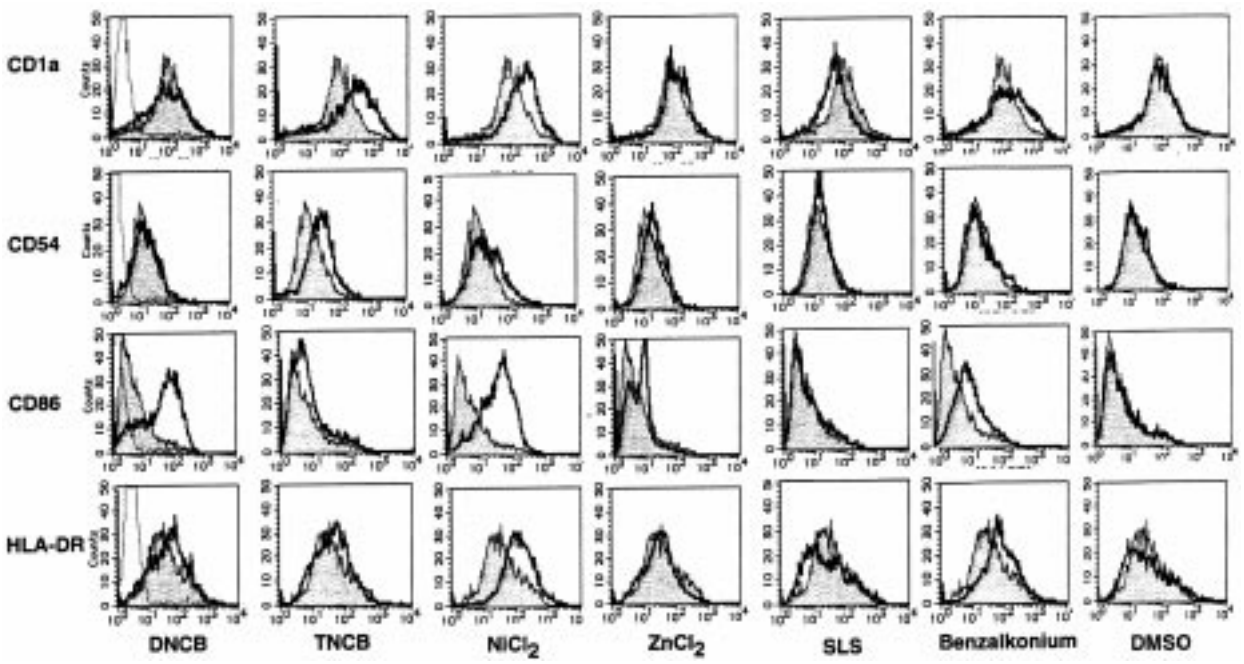

Figure 2. Treatment with $NiCl_2$ or DNCB augments the expression of CD86 on cultured monocyte-derived DC. CD1a+ DC were cultured with or without several chemicals for 2 d. Their surface phenotypes were analyzed by flow cytometry. Data are expressed in the form of a fluorescence histogram overlay depicting the staining of cells treated with specific chemicals (thick solid line), that without any chemicals (shaded curve), and the negative control staining (dotted line). (Reproduced with permission from Aiba *et al*, 1997.)

chemicals, and we suggest that DC, as cells involved in the innate immune system, play a crucial role in the immunity to simple chemicals.

MATERIALS AND METHODS

Media and reagents The medium used throughout the study was RPMI-1640 including 25 mM N-2-hydroxyethylpiperazine-N1–2-ethanesulfonic acid buffer solution (HEPES) (Sigma, St. Louis, MO) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin, streptomycin and fungizone antibiotic solution (Sigma), and 10% fetal calf serum (Summit Biotechnology) (complete medium). The following chemicals were used for stimulation of DC: nickel chloride ($NiCl_2$), cobalt chloride ($CoCl_2$), manganese chloride ($MnCl_2$), zinc chloride ($ZnCl_2$), and sodium lauryl sulfate (SLS) from Sigma, 2,4-dinitrochlorobenzene (DNCB) and cupric chloride from Wako Pure Chemicals (Osaka, Japan), 2,4,6-trinitrochlorobenzene (TNCB) from Tokyo Kasei (Tokyo, Japan), and 10% benzalkonium chloride (BC) solution from Japan Pharmaceutical (Tokyo, Japan). We used the following monoclonal antibodies (MoAb) for immunostaining: FITC-conjugated anti-CD80, anti-CD86, and anti-CD40 Ab (PharMingen, San Diego, CA), FITC-conjugated anti-CD54 Ab (Ancell, Bayport, MN), FITC- and PE-conjugated anti-HLA-DR Ab (Becton-Dickinson, San Jose, CA), PE-conjugated anti-CD1a Ab (Coulter, Hialeah, FL), FITC and PE-conjugated isotype-matched mouse control Ab (IgG2a and IgG2b) (PharMingen). For examining the effects of cytokines on surface molecule expression by DC, we used rabbit anti-IL-1β Ab, anti-TNFα Ab (Genzyme, Cambridge, MA), and nonimmunized rabbit serum (Sigma). MACS colloidal supermagnetic microbeads conjugated with monoclonal antihuman CD14 Ab (CD14 microbeads) were purchased from Mitenyi Biotec (Sunnyvale, CA). Recombinant human GM-CSF (rhGM-CSF) was a gift from Kirin Brewery (Tokyo, Japan), and recombinant human IL-4 (rhIL-4) and anti-TNFα (rhTNFα) were purchased from Genzyme (Cambridge, MA). DNCB and TNCB were solubilized in dimethyl sulfoxide (DMSO) at a concentration of 1 M. The final concentration of DMSO used was always at less than 0.1%.

Culture of DC from the peripheral blood monocytes (PBMC) PBMC were obtained by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation of heparinized fresh leukocyte-enriched buffy coats from different donors. Purification of CD14+ monocytes was performed by using MACS CD14 microbeads and a magnetic cell separator, MACS (Mitenyi Biotec), according to the manufacturer's protocol. Before culturing, we examined the percentages of CD14+ cells in those preparations by flow cytometry, and used cell specimens containing more than 98% CD14+ cells in the following experiments.

These CD14+ monocytes (2×10^6 per ml) were cultured in the complete medium containing 50 ng rhGM-CSF per ml and 100 ng rhIL-4 per ml for 5 d. Before the following chemical treatment, the cultured cells were examined for the expression of CD1a by flow cytometry.

Chemical treatment of cultured DC Five days after the culture with GM-CSF and IL-4, the cultured cells were treated with various concentrations of $NiCl_2$, $CoCl_2$, $MnCl_2$, $CuCl_2$, $ZnCl_2$, SLS, BC, DNCB, or TNCB for 24 h

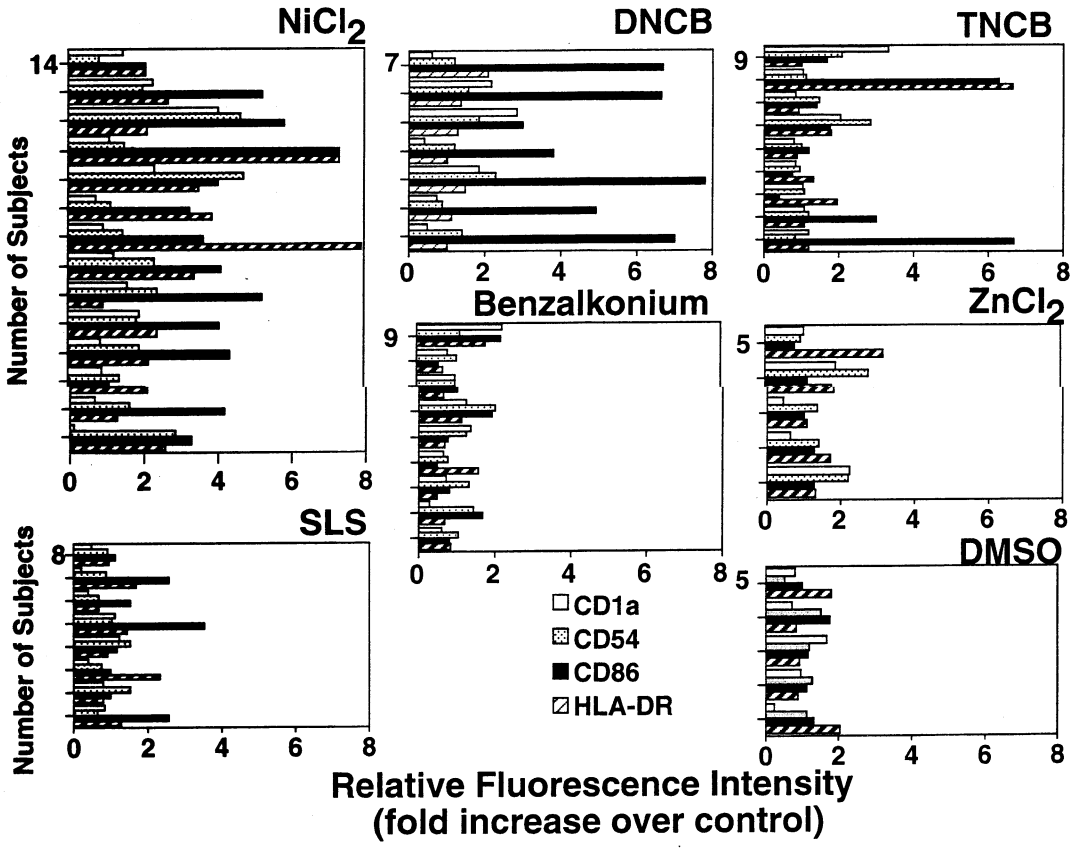

Figure 3. DC treated with $NiCl_2$ or DNCB augment their expression of CD86 or HLA-DR antigen. DC from five to 14 subjects were treated with several chemicals for 2 d, and their surface phenotypes were analyzed by flow cytometry. Relative fluorescence intensity (fold increase over control) was defined by the following formula:

Relative fluorescence intensity (fold increase over control) =
(Mean fluorescence intensity of DC treated with chemical) ÷ (Mean fluorescence intensity of nontreated DC)

Relative fluorescence intensities are expressed on DC treated with each chemical. (Reproduced with permission from Aiba *et al*, 1997.)

or 48 h. In some experiments, in addition to chemicals, an optimal concentration of the respective neutralizing Ab for IL-1β or for TNFα, or nonimmunized rabbit serum used as a control, was added to the culture.

Flow cytometry Twenty-four and 48 h after treatment with chemicals, the surface expression of HLA-DR and costimulatory molecules was analyzed by a FACScan using the CellQuest program (Becton-Dickinson). Dead cells were gated out with a final concentration of 0.5 mg propidium iodide solution per ml. In some experiments, we calculated relative fluorescence intensity (fold increase over control) by the following formula:

equation

Relative fluorescence intensity (fold increase over control) = (Mean fluorescence intensity of DC treated with chemicals) ÷ (Mean fluorescence intensity of nontreated DC)

ELISA for cytokine production The culture supernatant of the DC treated with various chemicals was recovered 24 h and 48 h after culture. The production of IL-1β, IL-6, and TNFα was measured by ELISA (R&D Systems, Minneapolis, MN), using 96 well microtiter plates, according to the manufacturer's instructions.

Detection of apoptosis For annexin V staining, DC at different time intervals after treatment with chemicals were washed with phosphate-buffered saline, stained for annexin V-FITC and propidium iodide (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's protocol, and analyzed by flow cytometry.

For the *in situ* end-labeling technique [terminal deoxynucleotide transferase (TdT) dUTP fluorescein nick end labeling (TUNEL)], an *in situ* apoptosis detection kit from Oncor (Gaithersburg, MD) was used according to the manufacturer's protocol, and analysis was done by flow cytometry.

Statistical analysis The statistical significance in the upregulation of mean fluorescence intensity of costimulatory molecule expression or HLA-DR antigen measured in flow cytometry, or in the amount of secreted cytokines, was analyzed using the Wilcoxon signed-rank test between nontreated DC and DC treated with chemicals.

RESULTS AND DISCUSSION

Augmented expression of CD86 and increased production of either IL-1β or TNFα by DC treated with chemicals *in vitro*

Although to induce T cell mediated immunity to protein antigens, so-called adjuvants are required to activate the innate immune system including DC, allergic contact hypersensitivity reaction does not require such help by adjuvants, and can be induced by simple application of haptens to the skin. When we consider the immunity to simple chemicals by analogy with the immunity to infectious agents, innate immunity should also play a role in it. DC or macrophages may discriminate innocuous and noxious chemicals and trigger T cell immunity. Indeed, by painting various chemicals on murine ears to examine the expression of class II MHC antigens and costimulatory molecules, we have demonstrated that Langerhans cells discriminate some chemicals. They respond to chemicals that are classified as haptens, i.e., dinitrochlorobenzene (DNCB), trinitrochlorobenzene (TNCB), dinitrofluorobenzene (DNFB), and cinnamic aldehyde, but not to primary irritants like benzalkonium, methyl salicylate, or phenol, which induce irritation of the skin, by augmenting their expression of class II MHC antigen and CD80 and CD86 (Aiba and Katz, 1990; Ozawa *et al*, 1996). Enk *et al* found that the increase in Langerhans cell-derived IL-1β mRNA signal strength is the first event in Langerhans

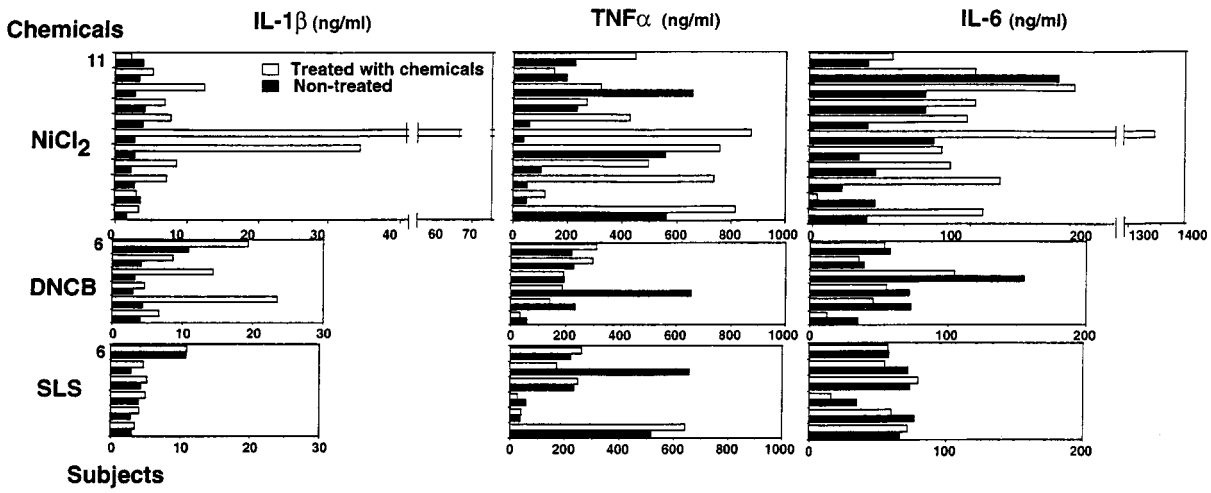

Figure 4. DC treated with $NiCl_2$ or DNCB increase their secretion of pro-inflammatory cytokines. DC from six to 11 subjects were treated with several chemicals for 2 d, and the culture supernatants were measured for IL-1β, TNFα, and IL-6 by ELISA. (Reproduced with permission from Aiba *et al*, 1997.)

cells after hapten painting (Enk and Katz, 1992a), and that the cutaneous injection of IL-1β results in increased expression of MHC class II Ag and enhanced antigen-presenting function by Langerhans cells (Enk *et al*, 1993). These data strongly suggest that DC recognize a difference in chemicals, and subsequently determine their activation by themselves. Because the skin also contains cells other than Langerhans cells, i.e., keratinocytes, mast cells, endothelial cells, and even T cells, however, it is indispensable to examine this interaction between DC and chemicals by using pure cultured DC, which can be kept in the immature state and made to mature with appropriate stimuli *in vitro*. The culture system of Langerhans cells is not suitable for this purpose because they can fully mature in an *in vitro* culture system without exogenous factors (Schuler and Steinman, 1985). Recently, Sallusto and Lanzavecchia (1994) and Romani *et al* (1994) have reported that immature DC can be obtained from a culture of peripheral blood monocytes with GM-CSF and IL-4.

Using this culture system, we examined the effects of the following chemicals on the expression of costimulatory molecules, CD1a, and HLA-DR antigen, by DC: $NiCl_2$, $ZnCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, SLS, BC, TNCB, and DNCB. **Figure 2** is a representative flow cytometric profile of DC from one subject, and **Fig 3** summarizes the effects of the chemicals on DC from different subjects. When we added these chemicals to the DC, we at first found that both $NiCl_2$ and DNCB, representative haptens, significantly increased the expression of CD54, CD86, and HLA-DR antigen on DC compared with that on nontreated controls or with that on DC treated with $ZnCl_2$, SLS, or BC, used as irritants. The increased expression of CD86 was accompanied by augmented expression of its mRNA demonstrated by reverse transcriptase-polymerase chain reaction (data not shown). In addition, the augmented expression of CD86 by DC treated with $NiCl_2$ or DNCB was accompanied by their increased secretion of either IL-1β or TNFα (**Fig 4**). These observations strongly suggest that DC respond to simple chemicals such as $NiCl_2$ or DNCB by changing their expression of costimulatory molecules or HLA-DR antigen and production of cytokines, subsequently altering their antigen-presenting function. Indeed, we observed that DC treated with $NiCl_2$ or DNCB significantly augmented their allogeneic T cell stimulatory function, which was dependent on their increased CD54 and CD86 expression (**Fig 5**). Although the numbers of subjects we examined were limited, we also found that $MnCl_2$ and $CuCl_2$ exerted effects on DC similar to those of $NiCl_2$ and $ZnCl_2$, respectively.

Different mechanisms of DC activation by $NiCl_2$ and by DNCB Next, to clarify the mechanism by which $NiCl_2$ or DNCB stimulated DC, we studied the effects of Ab for IL-1β and TNFα on the upregulation of CD86 expression by DC treated with $NiCl_2$ or DNCB. **Figure 6** shows representative data concerning the effects of anticytokine antibodies on CD86 expression by DC treated with

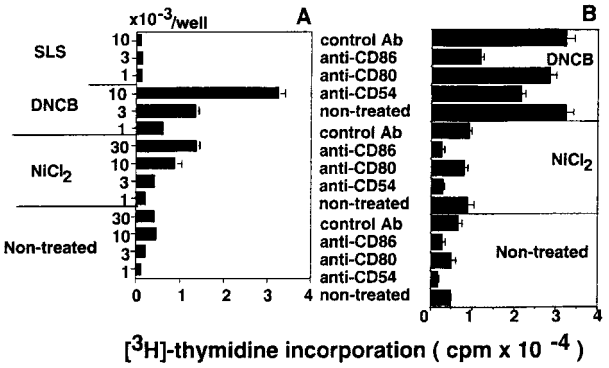

Figure 5. DC treated with $NiCl_2$ or DNCB augment allogeneic T cell stimulatory function of DC, which was suppressed by anticostimulatory molecule Ab. T cells (2×10^5 cells per well) were cocultured in 96 well flat-bottom microtiter plates with various numbers of DC with or without chemical treatment for 4 d at 37°C in a 5% CO_2 humidified atmosphere. During the last 16 h of culture, the cells were pulsed with 1 mCi [^{3}H]-TdR per well (*A*). To examine the role of CD54, CD80, or CD86 on allogeneic T cell stimulation by DC, 10 mg per ml of monoclonal anti-CD54 Ab, anti-CD80 Ab, or anti-CD86 Ab was added to the culture (*B*). (Reproduced with permission from Aiba, 1998.)

DNCB or $NiCl_2$. Statistical analysis of 15 different experiments demonstrated that the augmented expression of CD86 on DC treated with DNCB was suppressed by either the antibody for IL-1β ($p = 0.046$) or that for TNFα ($p = 0.0150$), whereas the augmentation induced by $NiCl_2$ was relatively insensitive to this antibody treatment. This observation suggests that different chemicals induce CD86 expression on DC via different mechanisms. That is, DNCB at first stimulates DC to secrete IL-1β or TNFα, which induces their CD86 expression. On the other hand, $NiCl_2$ may directly induce the augmentation of CD86 expression on DC. In fact, we found that, consistent with this observation, $NiCl_2$ and DNCB induced several different responses in DC. DC from a large number of subjects responded to $NiCl_2$ by increasing their expression of CD54 and HLA-DR antigen more strongly than DC treated with DNCB, whereas both chemicals induced similar magnitudes of upregulation of CD86. Although DC treated with $NiCl_2$ and DNCB increased their production of IL-1β, only those treated with $NiCl_2$ increased their production of IL-6 and TNFα. Regarding the effects of DNCB, these data are consistent with the observations by Enk *et al* (Enk and Katz, 1992a, b), which suggested the production of IL-1β by Langerhans cells treated with haptens and its crucial role in Langerhans cell maturation, although in our system TNFα seemed to exert more dramatic effects on Langerhans cell

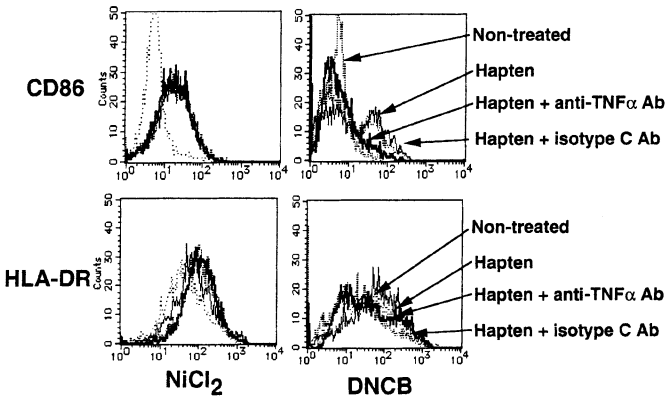

Figure 6. Anti-TNFα Ab abrogates the augmentation of CD86 expression on DC treated with DNCB, but not that on those treated with $NiCl_2$. Anti-TNFα Ab or isotype-matched control Ab was added to the culture of DC treated with $NiCl_2$ or DNCB. The effects were analyzed by flow cytometry. Data are expressed in the form of a fluorescence histogram overlay depicting the staining of DC treated with $NiCl_2$ or DNCB (thick solid line), that with anti-TNFα Ab (thin solid line), that with isotype-matched control Ab (broken line), and that of nontreated DC (dotted line).

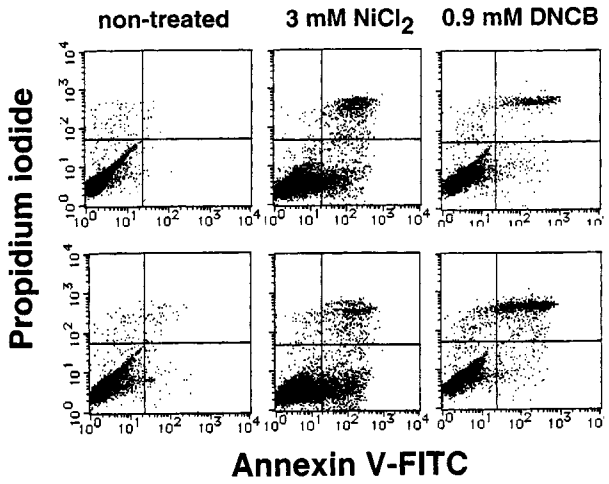

Figure 7. The treatment with $NiCl_2$ induces propidium iodide-negative (PI–) and annexin V-FITC-positive (annexin V+) DC. DC at different time intervals after treatment with chemicals were washed with phosphate-buffered saline, stained for annexin V-FITC and propidium iodide according to the manufacturer's protocol, and analyzed by flow cytometry.

activation than IL-1β. The effects of $NiCl_2$ on DC activation, however, were totally different from their observations. Although $NiCl_2$ increased the production of IL-1β, the augmented expression of CD86 on DC was not induced by the secreted IL-1β.

Apoptosis of DC To clarify the difference between the effects of $NiCl_2$ and DNCB on DC, apoptosis of DC was examined by two different procedures, i.e., annexin V staining and the TUNEL method. **Figure 7** presents representative flow cytometry of annexin V staining at 6 h after chemical application from five different experiments. $NiCl_2$ could induce PI-annexin V+ cells, putative apoptotic cells, in the concentration range from 1 to 10 mM, which was higher than the optimal concentration to induce DC maturation. On the other hand, DNCB or TNCB could not induce apoptosis in DC at any of the concentrations we examined. Consistent with these data, the TUNEL method also demonstrated TUNEL+ apoptotic cells in DC treated with $NiCl_2$, but not in those treated with DNCB, at 24 h after chemical application (**Fig 8**).

Possible cascade of DC recognition in innate immunity to simple chemicals These data clearly demonstrate that DC themselves recognize a difference in chemicals, and that those chemicals

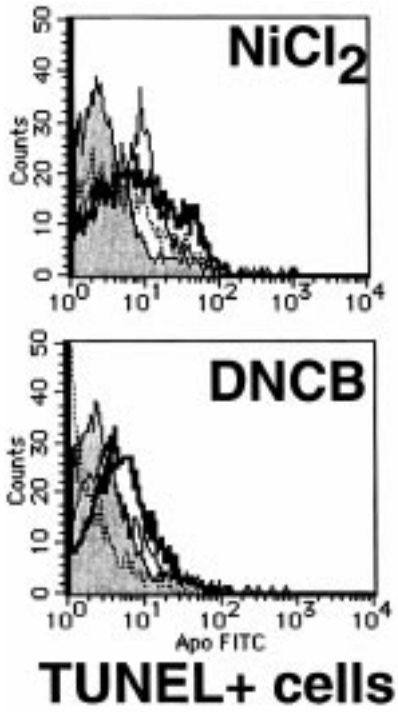

Figure 8. $NiCl_2$-induced TUNEL+ cells. Consistent with the data using annexin V staining, only the treatment with $NiCl_2$ resulted in TUNEL+ cells in DC. Data are expressed in the form of a fluorescence histogram overlay depicting the staining of DC treated with 9 mM $NiCl_2$, 900 mM DNCB, or 900 mM TNCB (thick solid line), with 3 mM $NiCl_2$, 300 mM DNCB, or 300 mM TNCB (thin solid line), with 1 mM $NiCl_2$, 100 mM DNCB, or 100 mM TNCB (broken line), and that of nontreated DC (shaded curve).

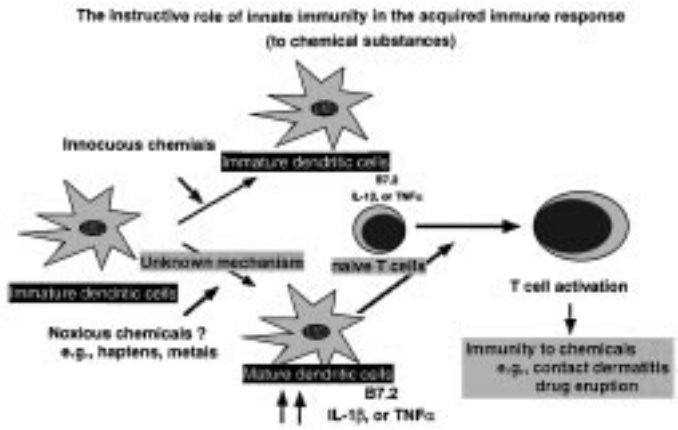

Figure 9. The instructive role of innate immunity played by DC in the acquired immune responses to simple chemicals. Immature DC are stimulated by certain simple chemicals and augment their expression of costimulatory molecules and secretion of pro-inflammatory cytokines. These mature DC can stimulate naïve T cells and induce specific immunity to chemicals. So far, however, the precise characteristics of the chemicals that can activate DC and the mechanism by which DC respond to the chemicals are unknown.

trigger their activation process and augment their expression of costimulatory molecules or class II MHC antigen and secretion of pro-inflammatory cytokines (**Fig 9**). In addition, some chemicals trigger their apoptosis. So far we do not know the precise reasons why the chemicals stimulated DC, although some relationship between the potential of chemicals to stimulate DC and that to sensitize animals is suggested. We do not know either why only certain chemicals can stimulate activation of DC. It is difficult to consider the possibility of the presence of receptors to recognize these chemicals. The chemicals that can activate DC do not necessarily share common chemical

characteristics. It is conceivable that chemicals induce a stress response that activates DC; however, because $NiCl_2$ and DNCB induce DC activation by different mechanisms and because different chemicals induce heterogeneous responses in the augmentation of costimulatory molecules or class II MHC antigen, that of cytokine secretion and induction of apoptosis, the activation of DC cannot be explained by a simple stress response. These chemicals may directly affect the intracellular signal transduction of DC.

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